

Identification of *PITX1* as a *TERT* Suppressor Gene Located on Human Chromosome 5[∇]

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Telomerase, a ribonucleoprotein enzyme that maintains telomere length, is crucial for cellular immortalization and cancer progression. Telomerase activity is attributed primarily to the expression of telomerase reverse transcriptase (*TERT*). Using microcell-mediated chromosome transfer (MMCT) into the mouse melanoma cell line B16F10, we previously found that human chromosome 5 carries a gene, or genes, that can negatively regulate *TERT* expression (H. Kugoh, K. Shigenami, K. Funaki, J. Barrett, and M. Oshimura, *Genes Chromosome Cancer* 36:37–47, 2003). To identify the gene responsible for the regulation of *TERT* transcription, we performed cDNA microarray analysis using parental B16F10 cells, telomerase-negative B16F10 microcell hybrids with a human chromosome 5 (B16F10MH5), and its revertant clones (MH5R) with reactivated telomerase. Here, we report the identification of *PITX1*, whose expression leads to the downregulation of mouse *tert* (*mtert*) transcription, as a *TERT* suppressor gene. Additionally, both human *TERT* (*hTERT*) and mouse *TERT* (*mtert*) promoter activity can be suppressed by *PITX1*. We show that three and one binding site within the *hTERT* and *mtert* promoters, respectively, that express a unique conserved region are responsible for the transcriptional activation of *TERT*. Furthermore, we showed that *PITX1* binds to the *TERT* promoter both *in vitro* and *in vivo*. Thus, *PITX1* suppresses *TERT* transcription through direct binding to the *TERT* promoter, which ultimately regulates telomerase activity.

Telomeres, which are specialized chromatin structures that are located at the end of eukaryotic chromosomes, are essential for the stability and integrity of chromosomes (2). Telomere erosion results from incomplete end replication. This erosion results in the loss of about 50 to 200 bp of telomeric DNA at each cell division and eventually leads to replicative senescence (6, 18). In contrast, telomerase, the ribonucleoprotein enzyme that maintains the telomere, is active in human germ cells, stem cells, and a majority of tumors (around 90%) and immortalized cell lines but not in somatic cells, suggesting that this enzyme activity contributes to an unlimited replicative potential and neoplastic transformation. This expression profile of telomerase activity further suggests that normal human cells contain a regulatory factor(s) that suppresses telomerase activity, and that this regulation is aberrant or missing following cellular immortalization (7). The telomerase holoenzyme consists of a protein catalytic subunit telomerase reverse transcriptase (*TERT*) and an integral RNA telomerase RNA component (*TERC*). The expression of *TERT*, but not *TERC*, depends on telomerase activity. Many studies have provided

evidence suggesting that ectopic *hTERT* expression into telomerase-negative normal cells, such as human fibroblasts and epithelial cells, restores telomerase activity and maintains telomeres, resulting in continuous cell division (1, 15, 16, 48). Moreover, the ectopic expression of *hTERT* in combination with activated oncogenes results in the tumorigenic conversion of normal human cells (17). These findings suggest that *TERT* is the principal component for the control of telomerase activity and is a key factor that facilitates cellular immortalization.

Microcell-mediated chromosome transfer (MMCT) is a powerful tool for (i) gene hunting, (ii) the elucidation of the essential function of products that are expressed from a specific chromosome with its endogenous physical system, and (iii) understanding the role of the components of chromosomes and understanding genome organization (12, 31). In addition, the functional analysis of cells that have undergone MMCT using a human chromosome truncated at specific sites enables the precise mapping and identification of genes involved in a particular cell function, such as telomerase regulation, during cellular aging and metastasis. This method also can be used to identify DNA repair genes whose mutation/deletion is responsible for various inherited genetic defects, to generate transgenic mice with introduced human chromosomes, and to establish animal models of human diseases (12, 31). Advances have been made in the technology of chromosome engineering using DNA targeting through homologous recombination. It has been reported that chicken pre-B DT40 cells, which are

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proficient for homologous recombination, can be used to improve the efficiency of DNA targeting (25). We and several other groups have shown that the introduction of chromosome 3, 5, 6, or 10 results in the complete repression of *hTERT* transcription in human tumor cells (10, 20, 32, 36, 37, 47). Furthermore, deletion analysis of chromosome 3 indicated three regions, 3p12-p21.1, 3p14.2-p21.1, and 3p21.3, at which *hTERT* regulatory factors might be located (10, 51). Additionally, a telomerase repressor gene(s) has been mapped to regions in 10p15.1 on chromosome 10 (32). We previously reported that the introduction of human chromosome 5 into B16F10 mouse melanoma cells inhibited the expression of *mtert*, but that late-passage clones exhibited the reactivation of telomerase activity. These results suggested that the loss of a gene(s) on this chromosome was responsible for telomerase reactivation, indicating that human chromosome 5 also contains a gene or genes that can regulate the expression of *mtert* in B16F10 cells (24). Recently, we have reported that *hTERT* has suppressed expression by the introduction of human chromosome 5 in human melanoma A2058 cells (39). Thus, these results suggested that normal human cells carry multiple telomerase repressor genes, and that these genes encode factors that regulate the activity of the enzyme.

The expression of *TERT* is accompanied by the regulation of the gene balance between positive, transcription-activating factors (c-Myc, Sp1, estrogen, and human papillomavirus E6) and negative, transcription-repressing factors (Mad1, WT1, Rb, MZF-2, CTCF, menin, transforming growth factor β [TGF β], BRCA1, and Tax) (13, 14, 28, 34, 35, 41, 50, 53–56). However, the mechanism that controls *TERT* gene expression has not yet been completely elucidated, suggesting the existence of additional regulatory mechanisms. Furthermore, although we have shown using MMCT analysis that human chromosome 5 carries a *TERT* suppressor gene(s), the target gene(s) has not yet been identified from this chromosome (24, 39). Therefore, the identification of novel telomerase regulatory factors is eagerly anticipated to uncover the signaling mechanism that underlies the development of tumors.

Paired-like homeodomain 1 (*PITX1*) was described originally as a bicoid-related homeobox transcription factor that is recruited to regulate the transcription of the pro-opiomelanocortin (*POMC*) gene in the adult pituitary, and it is involved in the differentiation of pituitary cells and in pituitary formation (26). *PITX1* is expressed exclusively in the hind limb, not in the forelimb, and plays a crucial role throughout the process of limb development, and it also determines the morphology of muscle, tendon, and bones of the hind limb (11, 44). The development of oral epithelium, the first branchial arch, and its derivatives also are known to require *PITX1* (27, 45). *PITX1* was later identified as a suppressor of *RAS* activity and tumorigenicity based on an RNA interference (RNAi) library screen that induced the knockdown of the function of a wide range of genes. The knockdown of *PITX1* transcription resulted in a transforming activity and a phenotype that was comparable to that of *RAS* overexpression. It has since been shown that *PITX1* inhibits tumorigenicity by the downregulation of the *RAS* pathway through *RAS* protein activator-like 1 (*RASAL1*), which is a member of the family of *RAS*-GTP-activating factors (*RAS*-GAPs) that negatively regulate *RAS* activity. *RASAL1* has been shown to be a direct target of *PITX1* (23).

However, the colony-forming ability of cells in which *RASAL1* is inhibited was significantly lower than that in which *PITX1* is inhibited, indicating that *RASAL1* is not the only target of *PITX1* that is responsible for the progression of tumorigenicity and for cell proliferation (23).

In this study, parental B16F10 cells, telomerase-negative B16F10 microcell hybrids with human chromosome 5 (B16F10MH5), and its revertant clones (MH5R) with reactivated telomerase were examined using cDNA microarray analysis to identify suppressor genes that carry telomerase repressor function. We report here on the identification of a possible candidate gene, *PITX1*, which is located on the human chromosome region 5q31 and which showed the inhibition of *TERT* transcription. *PITX1* directly binds to the *TERT* promoter via specific response elements, providing additional evidence that *PITX1* plays a significant role in the negative regulation of this promoter.

MATERIALS AND METHODS

Cell lines and cell culture. Mouse melanoma B16F10 cells and human melanoma A2058 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) at 37°C in a humidified incubator with 5% CO₂. The B16F10 microcell hybrids with an introduced human chromosome 5 were maintained in DMEM supplemented with 10% FBS and 800 μ g/ml G418 (Calbiochem, La Jolla, CA).

Microarray analysis. Total RNA samples were isolated from the cells and were reverse transcribed and labeled using one-cycle target labeling and control reagents as instructed by Affymetrix. The quantitative analyses of gene expression were performed using Affymetrix human genome U133A and mouse genome 430A chips by following the manufacturer's instructions (Affymetrix, Santa Clara, CA). The chips were scanned using an Affymetrix scanner 3000. Expression values were calculated using Affymetrix Gene Chip analysis software MAS 5.0. All samples were analyzed in duplicate to confirm the reproducibility of the results.

STS content analysis. Three sequence-tagged site (STS) markers, located on human chromosome 5, were assigned for PCR analysis. The sequences of the forward and reverse primers are the following: *bac51529T*, 5'-TCTCGTTATGCTTTCTCTCTAGAA-3' and 5'-GACAGTTGTGCTCTGGTAGTCCA-3'; *D5S2474*, 5'-AAGGCCAGTGGGAAAAG-3' and 5'-TGAAATTTTCTCTGTGACTCTACC-3'; and *D5S396*, 5'-CCCAGAATTAACATGGTGA-3' and 5'-TAGAGACAGTGTGCTGAGAGG-3'. The primers 5'-TCCACCAAGAGC TTCACCTT-3' and 5'-CGGTGAGGTTGTTGATGTTG-3' were used for *PITX1* detection. Thermocycling conditions were 30 cycles of denaturation for 1 min at 95°C, annealing for 30 s at 58°C, and extension for 1 min at 72°C. The amplified products were resolved on a 2% agarose gel.

RNA isolation, reverse transcriptase PCR, and qRT-PCR. Total cellular RNA was extracted using the RNeasy Minikit (Qiagen, Valencia, CA) and then was treated with RNase-free DNase I (Wako, Osaka, Japan) to remove residual genomic DNA. First-strand cDNA was synthesized from 2.3 μ g of total RNA using an oligo(dT)₁₅ primer (Roche, Indianapolis, IN) and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's recommendations. *PITX1* mRNA expression was examined using the primers 5'-TCCACCAAGAGCCTCACCTT-3' and 5'-CGGTGAGGTTGTTGATGTTG-3'. Thermocycling conditions were 30 cycles of denaturation for 30 s at 95°C, annealing for 45 s at 55°C, and extension for 30 s at 72°C for 30 cycles. The primers 5'-ATGTCACGGAGAGCACATTC-3' and 5'-CTGCAGATGGGCATGGCTA-3' were used for the detection of *mtert* mRNA expression, using 30 cycles of 30 s at 95°C, 45 s at 65°C, and 30 s at 72°C. The primers 5'-CGAGAGCAGACACCAGCAG-3' and 5'-TTTTACTCCCACAGCACCTC-3' were used for the detection of *hTERT* mRNA expression, using 28 cycles of 30 s at 95°C and 60 s at 68°C. *GAPDH* was similarly amplified as an internal control. The products were visualized following electrophoresis on a 2% agarose gel. The real-time quantitative reverse transcription-PCR (qRT-PCR) was performed using an Applied Biosystems 7300 thermal cycler and a SYBR green PCR kit (Applied Biosystems, Foster City, CA).

The amplification was performed using 40 cycles. The SYBR green fluorescence signal was monitored in each cycle for the reference and marker genes.

Assay of telomerase activity. *PITX1* expressing- and parent vector-transfected B16F10 cells were harvested 48 h after transfection. Telomerase activity was assayed by the stretch PCR method using the Telochaser telomerase assay kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

Gene silencing by RNA interference. *PITX1* expression was inhibited using Dharmacon ON-TARGETplus smart pool *PITX1* short interfering RNA (siRNA) (Latayette, CO). ON-TARGETplus nontargeting pool siRNA was used as a negative control. siRNAs were transfected by DharmaFECT 1 (Dharmacon), and cells were harvested 48 h after transfection and subjected to RT-PCR. The siRNA pool against *PITX1* includes CCAAACAGCACUCGUCGUU, GU GCAAGGGUGGCUACGUG, GGCUGAAGCGCGAGCGUAA, and CAAC GUACGCACUUCACAA.

Plasmid construction. Various lengths of *hTERT* and *mtert* promoter regions (shown in Fig. 4A), which included the transcription start site, were PCR amplified from genomic DNA and were inserted into the Acc65I/BglII-digested luciferase (Luc) reporter vector pGL3-basic (Promega, Madison, WI). For the mutation of the constructs, a 6-nucleotide deletion in the *PITX1* binding site was introduced using a PCR-based site-directed mutagenesis kit (Toyobo). The *PITX1* expression and parent vectors were purchased from Origene (Rockville, MD). All of the plasmids were confirmed to have the correct sequence by DNA sequencing.

Luciferase assay. The cells were plated in 12-well plates 24 h before transfection. Reporter plasmids (0.25 μ g) and effector plasmids (1.0 μ g) were transfected using the Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's protocol. pGL4.70-*renilla* (Promega) was cotransfected as an internal control. The cells were lysed 48 h after transfection and were subjected to a luciferase assay using the Picagene dual SeaPansy luminescence kit (Toyo Ink, Tokyo, Japan) according to standard protocols. All of the experiments were performed at least three times. Luciferase activity was calculated as the activity of the reporter constructs compared to the *Renilla* activity.

Western blot analysis. Lysates (20 μ g of protein) were subjected to SDS-PAGE using a 12% resolving gel and then were transferred to a polyvinylidene difluoride (PVDF) membrane (Pierce, Rockford, IL). The membrane was blocked and incubated with the primary antibody against *PITX1* (Abcam, Cambridge, MA) and the secondary antibody according to the manufacturer's instructions. Immunoreactive bands were visualized using the ECL detection system (Pierce).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared by the sequential lysis of the cells in hypotonic (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA and EGTA, 1 mM dithiothreitol [DTT], and protease inhibitors) and hypertonic (20 mM HEPES, 0.4 M NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA and EGTA, 1 mM DTT, and protease inhibitors) buffers. The probes used were the oligonucleotides 5'-CCTTTAAAAAGGCTTAGGGATCACTA-3' (for PRE1) and 5'-ATCACTAAGGGGATTCTAGAAGGC-3' (for PRE2).

The probes were labeled with [γ -³²P]ATP using the Megalabel kit (Takara Bio Inc., Shiga, Japan) and then were annealed with the corresponding antisense oligonucleotides. The binding reaction was performed using nuclear extracts (50 μ g protein) in 20 μ l binding buffer [8 mM HEPES, 2.5 mM Tris, 10% glycerol, 1 mM EDTA, 2.5 mM MgCl₂, 90 mM NaCl, 1 mM DTT, 0.5 μ g of poly(dI-dC) · poly(dI-dC), and protease inhibitors]. The labeled probe (20,000 cpm) then was added, and the solution was incubated at room temperature for 30 min and then kept on ice. The products subsequently were subjected to electrophoresis on a 4% polyacrylamide gel. The gel was dried and exposed to X-ray film. For competition analysis, a mutant oligonucleotide, 5'-CCTTTAAAAAGGCGGAG GGATCACTA-3' (for PRE1) or 5'-ATCACTAAGGGGCGTCTAGAAGAG C-3' (for PRE2), was added to the binding reaction mixture in a 500-fold excess compared to the amount of the probe and was incubated at room temperature prior to probe addition. For the supershift assay, the *PITX1* antibody or control IgG was added after the 30-min incubation with the probe, and the mixture then was incubated at room temperature for 10 min.

ChIP. The chromatin immunoprecipitation (ChIP) assay was performed according to a standard protocol. Briefly, to cross-link the DNA in chromatin to histones, the cells were incubated in 1% formaldehyde for 10 min at 37°C. After being washed with cold phosphate-buffered saline (PBS) containing protease inhibitors (Complete, EDTA free; Roche), the cells were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, with Complete). DNA then was broken into 200- to 1,000-bp fragments by sonication (Branson 250 microtip sonicator; Branson Ultrasonics, Danbury, CT). After dilution, resultant solutions containing the equivalent of 1 \times 10⁴ cells were used as an internal control (input). The remainder of the sample was immunoprecipitated using the *PITX1* antibody for 16 h at 4°C. Protein A-agarose (Upstate, Lake Placid, NY) then was used to collect the immunoprecipitated complexes, which were eluted using elution buffer (1% SDS, 0.1 M NaHCO₃, 10 mM DTT) after extensive

washing. The cross-link then was reversed by the addition of 5 M NaCl, which was followed by protease K treatment. DNA was recovered using phenol-chloroform extraction and ethanol precipitation and was used as a template for PCR to amplify the region of the *PITX1* binding sites in the *TERT* promoter. The forward and reverse PCR primers used were 5'-TTTCAAACCGCCCTTT-3' and 5'-CTGTACAGCTCGCTGGAG-3' for *hTERT* and 5'-CAGTTCAGGCC CATATCTCC-3' and 5'-TTTGGTGCCTTCAGCTTTCT-3' for *mtert*. PCR conditions were 10 min at 95°C, followed by 33 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 20 s, plus a final extension at 72°C for 1 min. The products were subjected to electrophoresis on a 2% agarose gel.

Immunohistochemistry. Immunohistological examination was performed on 16 surgically resected gastric adenocarcinomas. All specimens were extracted from the files of the Division of Organ Pathology, Faculty of Medicine, Tottori University, and affiliated teaching hospitals. Approval for the study was obtained from the Institutional Review Board of the Faculty of Medicine, Tottori University (approval number 283). All specimens were fixed with 10% formalin and embedded in paraffin. Three-micrometer-thick sections and a Histofine SAB-PO (R) immunohistochemical staining kit (Nichirei, Tokyo, Japan) were used for immunohistochemical analysis. Briefly, paraffin-embedded sections were dewaxed with xylene and gradually hydrated. Antigen retrieval was performed by autoclave in 10 mM citrate buffer (pH 6.0) for 10 min after endogenous peroxidase activity was blocked by immersing the slides in 0.3% hydrogen peroxide in methanol for 30 min. As primary antibodies, a rabbit polyclonal antibody raised against *PITX1* (1:200; Abcam, Cambridge, MA) was used. Immunoreactions were visualized with diaminobenzidine, and the sections were counterstained with hematoxylin.

RESULTS

Expression microarray analysis of B16F10 microcell hybrids for identification of telomerase repressor genes on chromosome 5. To gain insight into telomerase suppressor genes on human chromosomes, we previously generated mouse melanoma B16F10 microcell hybrids containing individual normal human chromosomes using MMCT. We found that human chromosome 5 carries a putative telomerase repressor gene (24).

The strategy that we used to identify this putative telomerase repressor gene(s) on human chromosome 5 is outlined in Fig. 1. We compared the results of a cDNA microarray analysis of parental B16F10 cells, a B16F10 microcell hybrid that was telomerase negative and contained a human chromosome 5 (MH5), and its revertant clones (MH5R) that exhibited the reactivation of telomerase due to extended culture passages. We first sought to identify genes that are differentially expressed by human chromosome 5 in B16F10MH5 clones compared to MH5R clones using Affymetrix human genome U133A gene chips, which encompass about 22,000 transcripts of known genes. In this study, a signal log₂ ratio (SLR) of 2 (fold change, ≥ 2) in duplicate experiments was used as an arbitrary cutoff for the determination of mRNA level changes. SLR indicates the absolute signal intensity that is calculated using the Affymetrix analysis software. The expression level of nine genes was increased more than 4-fold (SLR, ≥ 2) in the MH5 clone compared to that in control B16F10 cells or that in the MH5R clone. The MH5R clone showed expression levels similar to those of the control. Three of these nine genes localize to human chromosome 5. These genes, namely, the transforming growth factor beta-induced (*tgf β i*), elongation factor RNA polymerase II, 2 (*ell2*), and paired-like homeodomain 1 (*pitx1*) genes, also showed a lower expression in parental B16F10 cells than in MH5 clones when assessed using Affymetrix mouse genome 430A gene chips (Table 1).

To confirm the result of the microarray analysis, we inves-

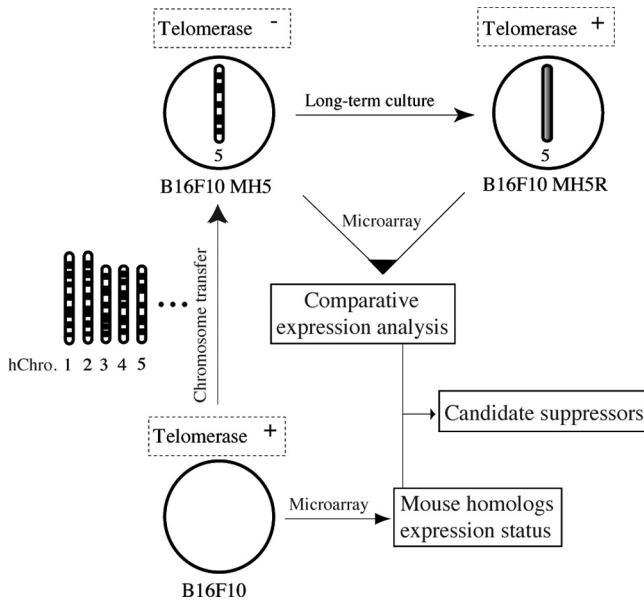


FIG. 1. Outline of the strategy used to identify negative regulators of telomerase. Normal human chromosomes were previously individually transferred into B16F10 cells. Only the introduction of human chromosome 5 (MH5) inhibited telomerase activity, which subsequently was reactivated during long-term culture (MH5R). Genes that were differentially expressed from human chromosome 5 between MH5 and MH5R were identified by cDNA microarray. Mouse homologs of these genes, which exhibited reduced expression in B16F10 cells, ultimately were chosen as candidate genes for further analysis.

tigated *PITX1*, *TGFβ1*, and *ELL2* mRNA expression profiles in MH5 and MH5R clones using qRT-PCR. The mRNAs of *PITX1*, *TGFβ1*, and *ELL2* were notably expressed in MH5 clones, in which *mtert* mRNA was barely detectable (Fig. 2A and B and data not shown). In contrast, the level of these mRNAs showed the reciprocal patterns in the parental cells and MH5R clones (Fig. 2A and B). Thus, the physiological expression of *PITX1*, *TGFβ1*, and *ELL2* mRNA negatively correlated with *mtert* mRNA expression in the B16F10 clones.

TGFβ1 encodes a secreted protein, transforming growth factor β (TGFβ), that is known to be a regulator of telomerase activity through the repression of the *hTERT* gene (57). *ELL2* encodes a member of the ELL family of RNA polymerase II elongation factors. The elongation stage of transcription plays an important role in the regulation of gene expression. It has been reported that the ELL family, containing *ELL2*, also regulates cell proliferation and cell survival (22). *PITX1* has

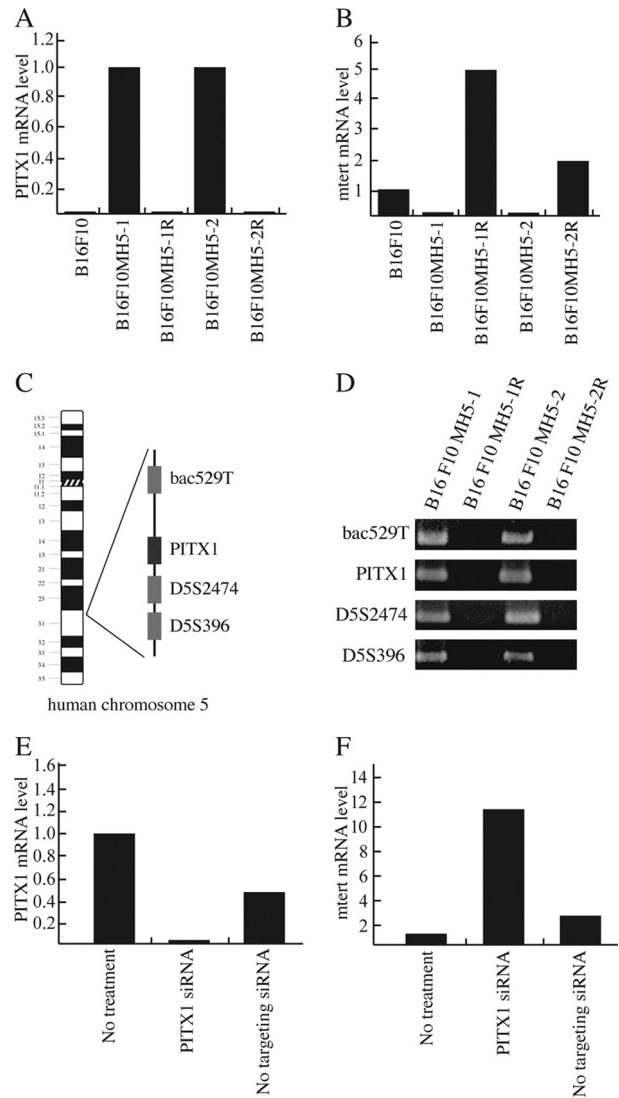


FIG. 2. Downregulation of *mtert* expression in MH5 clones is associated with *PITX1* expression. (A and B) Analysis of the mRNA expression of *PITX1* and *mtert* in microcell hybrids with human chromosome 5 (MH5) was performed using qRT-PCR. *GAPDH* was used as an internal control for each sample. MH5-1 and -2 showed strong repression of *mtert* expression, which was associated with the expression of *PITX1*. In contrast, revertant clones (MH5-1R and -2R) with telomerase activity showed reduced *PITX1* transcription. (C) Schematic diagram showing the location of the genomic loci of *PITX1* and flanking STS markers on human chromosome 5. (D) The chromosomal regions neighboring *PITX1* were not detected by genomic PCR in MH5R clones. (E and F) Depletion of *PITX1* by siRNA induces reactivation of *mtert* transcription in both MH5-1 and -2 clones in B16F10.

TABLE 1. Comparative expression of candidate telomerase suppressor genes analyzed using microarrays^a

Gene name	Gene accession no.	Probe set name	Exp 1				Exp 2					
			B16F10 signal	MH5-1 Signal	MH5-1R SLR	MH5-2 Signal	MH5-2R SLR					
<i>TGFBI</i>	NM_000358.1	201506_at	95.3 (A)	1,639.4 (A)	3.5 (I)	109.7 (A)	0.1 (NC)	92.4 (A)	1,287.4 (A)	2.6 (NC)	138.1 (A)	0.3 (NC)
<i>PITX1</i>	NM_002653.1	208502_s_at	145.7 (A)	1,616.5 (A)	2.7 (I)	243.6 (A)	0.1 (NC)	240.4 (A)	2,674.1 (A)	3.3 (I)	330.9 (A)	0.8 (NC)
<i>ELL2</i>	NM_012081.1	214446_at	198.2 (A)	2,818.8 (A)	3.6 (I)	784.2 (A)	1.5 (NC)	98.3 (A)	1,784.7 (A)	2.9 (I)	755.8 (A)	1.9 (I)

^a The change in signal intensity for a specific probe between B16F10 and MH5 or MH5R is shown as either increased (I) or not changed (NC). SLR is a comparison between signal intensities for B16F10 parental cells and telomerase-negative or reactivated MH5 clones. The detection algorithm used probe set intensities to assign an absent (A), present, or marginal call.

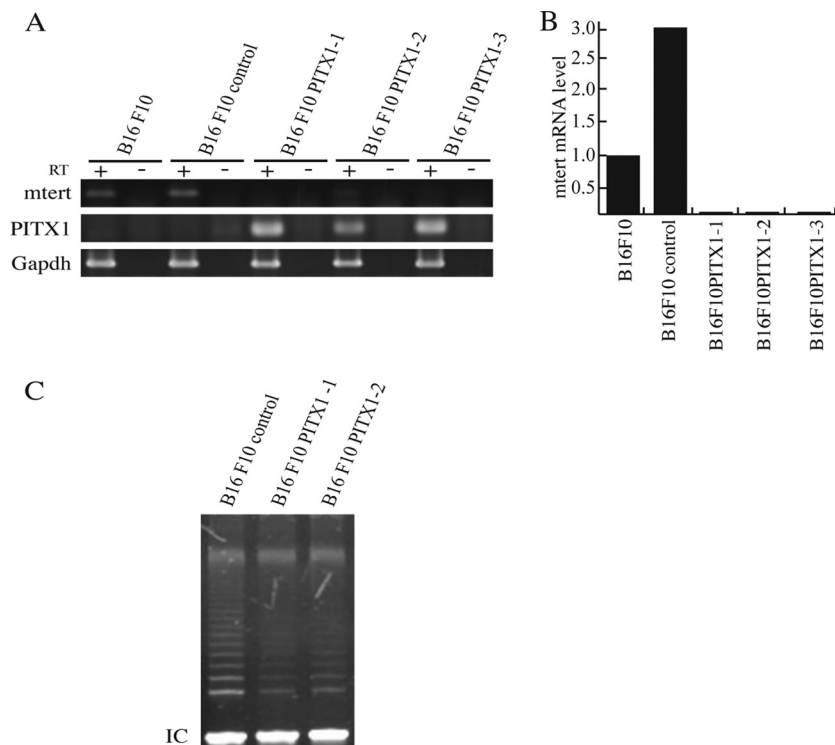


FIG. 3. Expression of *PITX1* inhibited *mtert* mRNA expression and telomerase activity in B16F10 cells. (A and B) RNA was prepared from B16F10 cells transfected with a *PITX1* expression vector or with control vector (*pcDNA3.1*) and was analyzed for *mtert* and *PITX1* mRNA expression using RT-PCR and qRT-PCR. (C) *PITX1* transfection resulted in depleted telomerase activity as assessed using the TeloChaser kit. IC is the internal control provided in the kit.

been reported to inhibit *RAS* activity and tumorigenicity, and it also induces the activation of the *p53* tumor suppressor gene in human breast cancer by directly binding to its promoter region (23, 29). These results suggest that the screening of the expression profiles of telomerase-positive and -negative MH clones of this study is an effective strategy for the identification of novel telomerase regulatory factors that are associated with cell proliferation.

Since little is known about the role of *PITX1* in the maintenance of telomerase enzyme activity, including the regulation of *TERT* transcription, we focused our study on *PITX1*.

Downregulation of *mtert* expression in MH5 clones is associated with *PITX1* expression profiles. We further compared the state of the genomic loci containing *PITX1* on the introduced human chromosome 5 in MH5 and MH5R clones by PCR analysis, using three chromosome 5-specific STS markers and a *PITX1*-specific primer (Fig. 2C). All of the loci examined (*bac51529T*, *D5S2474*, *D5S396*, and *PITX1*) were commonly deleted in MH5R, but not in MH5, clones (Fig. 2D). This result indicated that the observed decrease in *PITX1* mRNA expression is a result of either a deletion or a rearrangement of the genomic region harboring *PITX1* in the introduced human chromosome 5.

To further examine *mtert* suppression effects by *PITX1* under physiological conditions, we knocked down *PITX1* expression in MH5 clones that introduced human chromosome 5 in mouse melanoma B16F10 cells and human melanoma A2058 cells using RNAi. As shown in Fig. 2E and F, the depletion of *PITX1* leads to the reactivation of *TERT* RNA expression in

both B16F10MH5 and A2058MH5 clones, suggesting that *PITX1* is a negative regulator of *TERT* transcription in both human and mouse (data not shown).

Expression of *PITX1* inhibits *mtert* transcription and telomerase enzymatic activity. To investigate the effect of *PITX1* expression on telomerase activity, we transfected B16F10 cells with an expression vector (*pcDNA3.1-PITX1*) containing the full-length *PITX1* coding sequence or with a negative-control empty vector and assayed the subsequent mRNA expression of *mtert* using RT-PCR and qRT-PCR. As shown in Fig. 3A and B, the expression of *PITX1* resulted in a dramatic reduction in *mtert* mRNA expression. In contrast, *mtert* mRNA expression was similar to that of parental B16F10 cells in cells transfected with the empty vector. These findings suggested that *PITX1* can somehow suppress *mtert* mRNA expression in B16F10 cells.

The level of *TERT* transcription most commonly is regulated in a telomerase enzyme activity-dependent manner. Therefore, we next measured the effect of *PITX1* transfection on telomerase activity using a telomerase activity detection kit (TeloChaser) that is based on the stretch PCR method. B16F10 cells transfected with *PITX1* showed significantly reduced telomerase activities compared to that of the corresponding parental cells (Fig. 3C). These results suggest that the mechanism by which *PITX1* suppresses *mtert* mRNA expression involves the inhibition of telomerase activity.

***PITX1* inhibits telomerase enzymatic activity through the regulation of *TERT* promoter activity.** To investigate whether *PITX1* inhibits *TERT* mRNA expression through the modula-

tion of *TERT* promoter activity, we constructed a mouse *tert* promoter-luciferase reporter plasmid (pGL3) containing a 1,955-bp fragment (mtert1955) from within the *mtert* promoter region or various truncated fragments (mtert1747, mtert824, mtert356, and mtert155) of the 5' region of the *tert* gene (Fig. 4A). We then examined the effect of transient *PITX1* cotransfection with these pGL3-mtert-Luc reporters, or with an empty pcDNA3 control vector, into mouse B16F10 cells on the transcriptional activity of these *tert* promoters by the measurement of Luc reporter activity (Fig. 4A). The expression of *PITX1*, but not parental vector transfection, decreased the promoter activity of mtert1955 and mtert1747 by 60% ($P < 0.01$) and that of mtert824, mtert356, and mtert155 by 40% (Fig. 4A). These data suggest that *PITX1* directly regulates *mtert* transcription, and that the *mtert* promoter region between -1747 and -824 is critical for *PITX1* suppression.

Genomatrix and sequence analysis has identified potential *PITX1* binding sites within the *PITX1* transcription factor regulatory element (PRE) as the sequences TAA(T/G)CC [GG(C/A)TTA in reverse], AAATCC (GGATTT in reverse), and TAATCC (GGATTA in reverse) (23). In this report, these sites are designated PRE1, PRE2, and PRE3, respectively (Fig. 4B). We identified two PRE2 sites ($-1850/-1845$ and $-273/-268$) and one PRE3 site ($-1100/-1095$) in the *mtert* promoter region and one each of the PRE1 ($-1366/-1361$), PRE2 ($-1344/-1339$), and PRE3 ($-1322/-1317$) sites in the *hTERT* promoter region (Fig. 4B).

The Luc reporter experiments using fragments of the *mtert* promoter suggested that the PRE3 present in the -1100 region of the *mtert* promoter is responsible for the suppressive effect of *PITX1*. To further examine this possibility, we first generated *mtert* mutant promoter plasmids (mtert1955mut3) in which the sequence of PRE3 in mtert1955 was deleted (Fig. 4C). This mutation of PRE3 reduced *mtert* promoter activity to 20% of that of parental mtert1955 and gave the same level of promoter activity as that of the truncated fragments mtert824, mtert356, and mtert155 (Fig. 4A and D). We next constructed mutant mtert promoter reporter plasmids in which either one PRE2 site (mtert1955mut2L), two PRE2 sites (mtert1955mut2), or one PRE3 site (mtert1955mut3) was deleted. The reporter plasmid containing the PRE3 mutation showed a significant recovery of *mtert* transcriptional activity compared to that of the PRE2 mutation (mtert1955mut2L and mut2) and wild-type reporter ($P < 0.01$) (Fig. 4C and D). In addition, the result of suppression effect by PRE mutation was similar to that of the short type of *mtert* reporter plasmids (mtert1747) (data not shown). Although we cannot rule out the possibility that another mechanism(s) of *mtert* transcription also is involved, PRE3 in the *mtert* promoter may play a crucial role in the regulation of *mtert* transcription. Thus, this result suggested that *PITX1* can inhibit *mtert* transcription through the *mtert* promoter.

We next determined whether the *hTERT* promoter is regulated by *PITX1* in a manner similar to that of the *mtert* promoter. Sequence analysis indicated that only a region of approximately 300 bp is highly conserved between the *hTERT* and the *mtert* promoters (Fig. 4E). Interestingly, all three PRE sites of the *hTERT* promoter lie within this conserved region, suggesting that this region also plays a significant role in the regulation of *hTERT* transcription. To test this prediction, two luciferase reporter plasmids were prepared in which the pro-

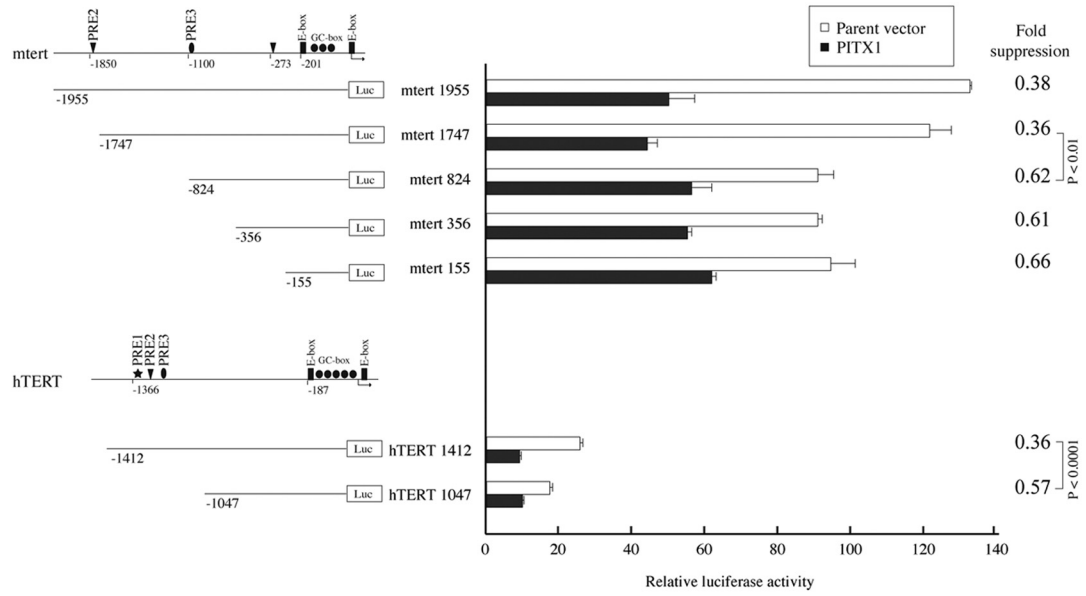
motor activity of 1.4 (hTERT1412)- and 1.0 (hTERT1047)-kbp fragments of the *hTERT* promoter, with and without PRE sites, respectively (Fig. 4A), was examined following the cotransfection of *PITX1* in B16F10 cells. Luciferase assays indicated that cotransfected *PITX1* decreased the promoter activity of hTERT1412 by 60% ($P = 0.0001$) and that of hTERT1047 by 40% compared to that of the parent vector (Fig. 4A). This result indicated that *PITX1* also affects *hTERT* transcriptional activity through PRE sites.

To confirm that the decreased *hTERT* promoter activity induced by *PITX1* in mouse cells also could occur in human cells, the luciferase reporter assays using transiently cotransfected hTERT1412 or hTERT1047 and *PITX1* were repeated using a human melanoma cell line, A2058, which also lacks endogenous *PITX1* expression (data not shown). As shown in Fig. 5A, *PITX1* also induced a decrease in hTERT1412 promoter activity in these cells that was 1.9-fold less ($P = 0.01$) than the activity of the hTERT1047 promoter, indicating that *PITX1* also regulates *hTERT* promoter activity in humans. To further analyze the *PITX1* modulation of the *hTERT* promoter in A2058 cells, we constructed, and similarly analyzed, mutant *hTERT* promoter reporter plasmids in which either PRE1 (hTERT1412mut1), PRE2 (hTERT1412mut2), or PRE3 (hTERT1412mut3) was disrupted by changing the sequence from 5'-GGCTTA-3' to 5'-AGCTTA-3', from 5'-GGATTT-3' to 5'-AAATTT-3', or from 5'-GGATTT-3' to 5'-AGATGT-3', respectively (Fig. 5B). The mutation of any of these PREs resulted in a 2- to 2.1-fold increase in *hTERT* promoter activity compared to that of the parental hTERT1412 (Fig. 5C), indicating that these PREs are essential for the *PITX1* modulation of *hTERT* promoter activity. These findings suggest that the suppression of *hTERT* promoter activity by *PITX1* is dependent exclusively on *PITX1* consensus binding sites in the promoter. Therefore, these PRE sites may play a crucial role in the regulation of *hTERT* promoter activity.

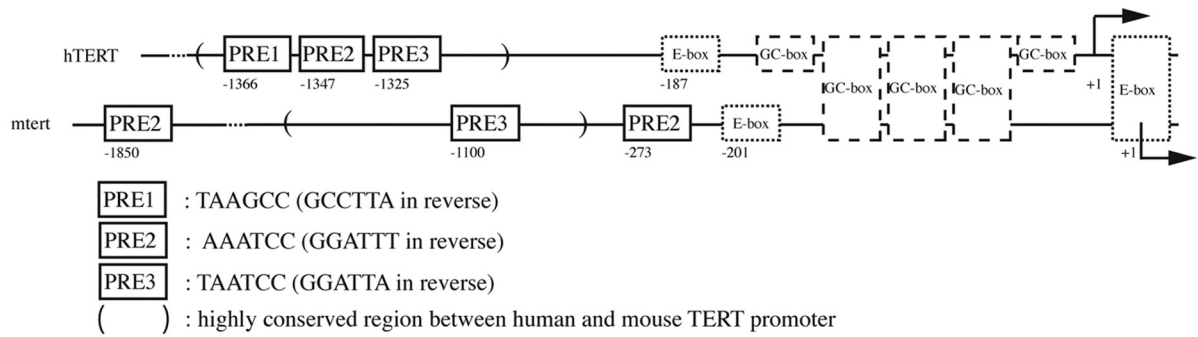
***PITX1* directly binds to the *TERT* promoter *in vitro* and *in vivo*.** To determine whether *PITX1* directly binds to the *hTERT* promoter, we performed EMSA using nuclear extracts prepared from parental B16F10 and *PITX1*-transfected B16F10 cells. Western blotting confirmed that the *PITX1* protein was expressed in *PITX1*-transfected B16F10 cells (Fig. 6A). We then assayed the binding of *PITX1* to DNA probes containing either PRE1 or PRE2 of the *hTERT* promoter using an EMSA (Fig. 6B and C, respectively). Each probe formed a complex with *PITX1*. Although some background signals were detected in the parental B16F10 lysate (Fig. 6B and C, lane 2), *PITX1* did form complexes with the labeled probes PRE1 and PRE2, which were abrogated by excess unlabeled wild-type probe but not by mutated probes (Fig. 6B and C, lanes 5 to 7). To verify the specificity of the *PITX1*-DNA interaction, we performed a supershift EMSA using the *PITX1* antibody. As shown in Fig. 6D, the complexes (P) formed by *PITX1* with the PRE1 (left) or PRE2 (right) probe were attenuated by a specific anti-*PITX1* antibody but not by control IgG. These results indicate that *PITX1* directly binds to both PRE1 and PRE2 within the *hTERT* promoter *in vitro*.

To determine whether *PITX1* could bind to the PRE regions of the *hTERT* or *mtert* promoter within cells, we carried out ChIP assays using the human and mouse melanoma cell lines, A2058 and B16F10, respectively, that were transiently trans-

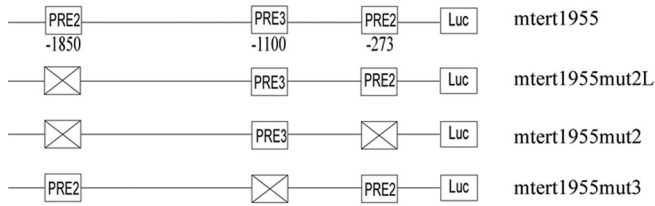
A



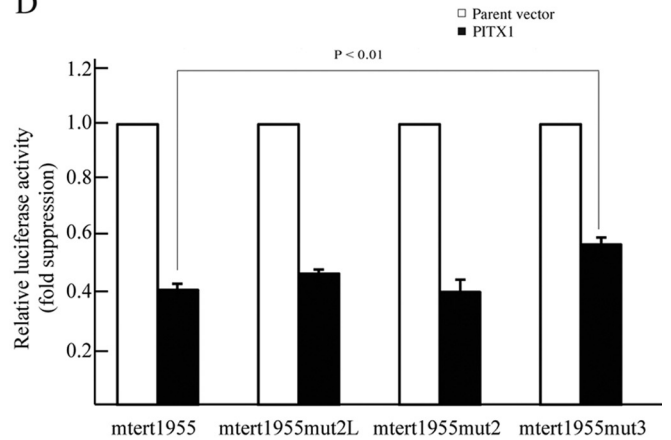
B



C



D



E

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hTERT -1495 TGTTTCTATGTTGGCTTCTCTGCAGAGAACCAGTGTAAAGCTACAACCTTAACCTTTGTTGGAACAAATTTCCAAACC--GCCCTTTGCCCTA-GTGGC -1399
***** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **   ** ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **
mtert -1267 TGTTTCTATGCTGGTTTCTTTG--GGAACTACACTAAGGT--AGCTTCA---TTGTTGGCATAAATTTCTCAGTTCAGGCCATATCTCCTAAGTAGC -1175

                PRE1                PRE2                PRE3
-1398 AGAGA----CAATTCACAACACAGCCCTTTAAAAAGGCTTAGGGATCACTAAGGGGATTTCTAGAAGAGCGACCCGTAATTCCTAAGTATTACAAGAGC -1303
*** ** ** ** ** ** ** ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **
-1174 AGAACTAAGCAAATCTCAAACA-AACCCCTCAAAGGACTGATG-TCCACTAAGCGGACTTCTAAATAGC-TCCTGTAATCCTGAGCATTACAAGGC- -1079
                PRE3

-1304 AGGCTAACCTCCAGCGAGCGTGACAGCCAGGGAGGGTGCAGAGGCTGTTCAAATGCTAGCTCCATAAATA-AAGCAATTTCCCGGCAGTTTCTGAAA -1204
** ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **
-1078 -GGCAGACCTCTATAAGGAGTAAATAT---GAAAACGCG---CCTGTTCAAATGCTAGGTCGGTGGATAGAAGCAATTTCTCAGAAAAG---CTGAAG -989

-1205 GTAGGAAAGGTTACATTTAAGGTTGCGTTTGTAGCATTTCAGTGTGTTCCGACCTCAGCTACAG -1138 hTERT
** ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **   ** ** **
-988 G-----CACCAAAGGTTATATTTGTTAGCATTTCAGTGTGTTCCAACTCAGCTACAG -935 mtert
  
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ected with *PITX1* in B16F10 and A2058 cells and MH5 clones. The promoter region that contains PRE1, PRE2, and PRE3 sites in *hTERT* and the PRE3 site in *mtert* was specifically amplified by PCR from chromatin that was precipitated with the anti-*PITX1* antibody from all cell types (Fig. 7 and data not shown). These findings suggest that *PITX1* can directly bind to the *TERT* promoter *in vivo*.

Expression of *PITX1* protein in human resected gastric normal mucosa and carcinoma tissue specimens. It has been reported that *PITX1* expression is reduced in various types of human cancers, including gastric, bladder, and colon cancers (4, 5). To provide further direct evidence that *PITX1* expression levels are related to human cancer, we examined *PITX1* expression in 16 surgically resected gastric adenocarcinoma specimens by immunohistochemistry. We found that *PITX1* was expressed on the sections of nontumoral gastric mucosae of all 16 specimens. In contrast, 70% of samples (11/16) showed *PITX1*-negative staining on the carcinoma sections (Fig. 8). Thus, these findings strongly support the hypothesis that the downregulation of *TERT* by *PITX1* plays a crucial role in the multistep process of neoplastic development.

DISCUSSION

Using MMCT into the mouse melanoma cell line B16F10, we previously provided evidence to suggest that many tumor suppressor genes are involved in the multistep process of neoplastic development (24). B16F10 microcell hybrids with an introduced normal human chromosome 5 displayed the inhibition of telomerase activity due to a reduction in the level of *mtert* mRNA. We have reported recently that the introduction of human chromosome 5 showed a remarkable decrease in the growth rate and eventually cellular senescence in human A2058 melanoma cells. Moreover, this phenomenon also was accompanied by a reduction of *hTERT* expression and telomerase activity (39). In this study, using comparative microarray analyses and assays of promoter function and binding, we identified *PITX1* as a telomerase repressor gene on human chromosome 5 that directly regulates *hTERT* transcription. *PITX1* is located at the chromosome region 5q31 (9). The loss of a specific region of chromosome 5q31 has been observed in several types of human cancers, such as acute monocytic leukemia (AML) and esophageal and breast cancer cells (3, 21, 40). We explored the *PITX1* tumor expression profile using the

ONCOMINE cancer microarray database (42). The results showed that *PITX1* expression is reduced in Barrett's esophagus and is significantly reduced during progression to Barrett's-associated adenocarcinoma (30). This phenomenon also is observed in lung, gastric, and colon cancer cells as well as tumor tissues of prostate and bladder but not in the respective normal tissues (4, 5, 23, 40, 46). In addition, the disappearance of *PITX1* was observed in 70% of gastric adenocarcinoma specimens (Fig. 8). Moreover, the suppression of *PITX1* expression by siRNA results in the reactivation of endogenous *TERT* expression in MH5 clones (Fig. 2E and F). Thus, these findings provide evidence that dysfunctions of the *PITX1* gene play an important role in the development of various types of human cancers.

Although a putative telomerase repressor gene was mapped to human chromosome bands 5p11 to 5p13 by a combination of functional analysis using the transfer of subchromosomal transferable fragments of chromosome 5 into B16F10 cells and by the deletion mapping of revertant clones with reactivated telomerase activity, the *PITX1* gene that we found to be a negative regulatory factor for *hTERT* in this study was located in the chromosome 5q31 region (38). It is possible that this large discrepancy between the mapping information for a candidate gene and the actual *PITX1* chromosomal localization is due to the scale of the screen used to identify the responsible gene. We used only 21 chromosome 5-specific STS markers to identify the location of the telomerase repressor gene. However, we do not rule out the possibility that there is another telomerase repressor gene that is located in the 5p11 to 5p13 region.

The overexpression of *PITX1* resulted in *p53*-dependent cell cycle arrest and apoptosis in the human mammary carcinoma cell line MCF-7 (29). *PITX1* has been shown to activate *p53* transcription via direct binding to the *p53* promoter. Moreover, *p21* and the placental transforming growth factor β gene (*PTGF β*), known as *p53* downstream target genes, were modulated by *PITX1* in a gene dosage-dependent manner, and *PTGF β* is known to inhibit tumor cell growth in the *TGF β* signaling pathway (29, 50). In addition, since *p53* can physically interact *in vivo* with *Smad2*, which is required for *TGF β* responses (8), it is likely that the modulation of the transcriptional activation of *p53* by *PITX1* enhances the activity of the *TGF β* signaling pathway by a number of mechanisms. However, *PITX1* still can induce apoptosis even in *p53*-depleted

FIG. 4. Luciferase assay of various truncated *hTERT* and *mtert* promoters in *PITX1*- and control vector-transfected B16F10 cells. (A) The schematic drawing (left) shows the position of the truncation site of each of the reporter plasmids. The firefly luciferase activity was standardized using *Renilla reniformis* luciferase activity from cotransfected pGL4.70. The promoter region that contains PREs significantly inhibits, and is therefore crucial for, *TERT* transcriptional activity in both humans and mice. Error bars represent standard deviations from three experiments. Statistical analysis (*t* test) using SigmaPlot 2000 and indicating a significant difference is shown (*P* values). (B) Comparison of transcription factor binding sequences in the *hTERT* and *mtert* promoters. The proximal promoter region of *hTERT* is partially similar to that of *mtert*, containing both E and GC boxes. The transcription start site is indicated by an arrow. Candidate *PITX1* binding sequences are indicated as *PITX1* response elements (PRE1, PRE2, and PRE3). (C) Schematic diagram showing the luciferase reporter plasmids that encode wild-type and PRE3-mutated versions of a fragment of the *mtert* promoter region. The crossed box represents mutation. (D) *PITX1* expression vectors were cotransfected with the *mtert* reporter plasmids shown in panel C and *mtert* 824, which lacks PRE3, and promoter activity was assayed using a luciferase assay. The firefly luciferase activity was standardized using *Renilla reniformis* luciferase activity from cotransfected pGL4.70. The transfection of control vector was used as a suppression control to normalize the effect of *PITX1* transfection. The transcriptional activity of *mtert* was suppressed by the wild-type reporter compared to that of the mutated reporters. Error bars represent the standard deviations from three experiments. Statistical analysis (*t* test) using SigmaPlot 2000 and indicating a significant difference is shown (*P* values). (E) The sequences within the *TERT* promoter conserved between human and mouse. Three and one PRE sites of the *hTERT* and *mtERT* promoters lie within this conserved region.

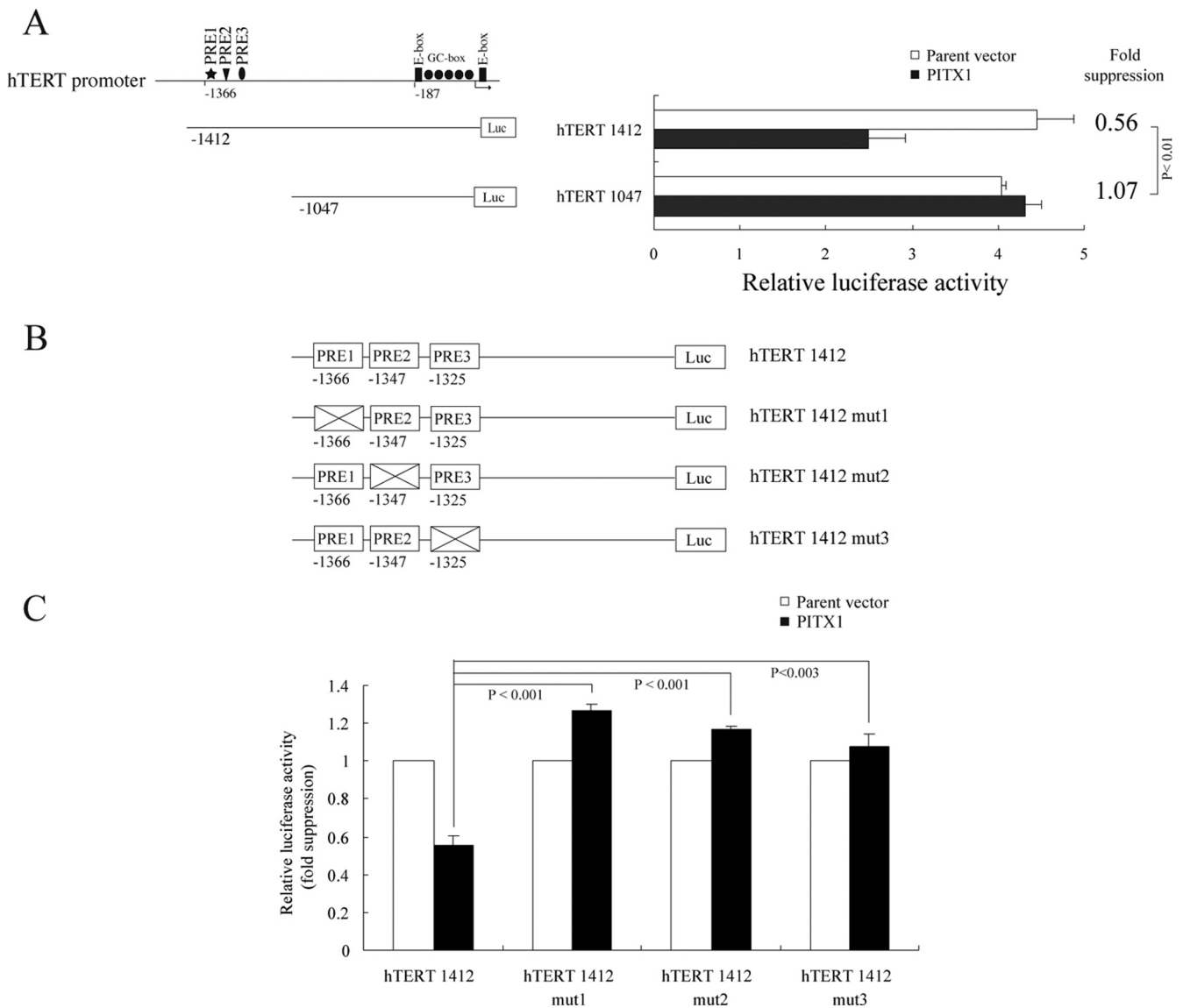


FIG. 5. Suppression of the transcriptional activity of the *hTERT* promoter by *PITX1*. (A) *PITX1* expression vectors were cotransfected into the human melanoma cell line A2058 with reporter plasmids containing a wild-type (*hTERT1412*) or a truncated (*hTERT1047*) *hTERT* promoter region in which the PRE site in the *hTERT* promoter region is eliminated. The resulting firefly luciferase activity was standardized using the *Renilla reniformis* luciferase activity from cotransfected pGL4.70. The transcriptional activity in *hTERT1412* indicated suppression effects that were approximately 2-fold greater than those of *hTERT1047*. Error bars represent the standard deviations from three experiments. Statistical analysis (*t* test) using SigmaPlot 2000 and indicating a significant difference is shown (*P* values). (B) Schematic diagram showing the luciferase reporter plasmids that carry wild and mutated versions of the *mtert* promoter region. The crossed box represents mutation. (C) *PITX1* expression vectors were cotransfected with reporter plasmids containing the wild-type or PRE-mutated versions of the *hTERT* promoter. The transcriptional activity of *hTERT* was suppressed by the wild-type reporter compared to that of the mutated versions. Error bars represent the standard deviations from three experiments. Statistical analysis (*t* test) using SigmaPlot 2000 and indicating a significant difference is shown (*P* values).

MCF-7 cells (29), suggesting the possibility that *hTERT* inhibition by *PITX1* is not the only mechanism by which *p53* causes telomerase inhibition.

Using MMCT, we and other investigators found that telomerase repressor genes are located on at least four different human chromosomes, 3, 5, 6, and 10 (10, 20, 24, 32, 36, 47), suggesting that there are multiple telomerase-dependent pathways for the regulation of cellular senescence (37). It is likely that defects in these pathways are required for complete cellular immortalization and to predispose cells to neoplastic transformation.

There are some significant differences in telomere regulation between human and mouse biology. In particular, mouse telomeres are, on average, much longer than those in humans. Moreover, the expression of *hTERT* is more tightly regulated than that of *mtert* in normal somatic cells, although the expression of both genes is strongly upregulated in tumors. A transgenic mouse carrying a *lacZ* gene driven by the *hTERT* promoter has shown that the activity of the *hTERT* promoter in normal mouse tissues recapitulates the expression of the *hTERT* gene in normal human tissues (43). Furthermore, the

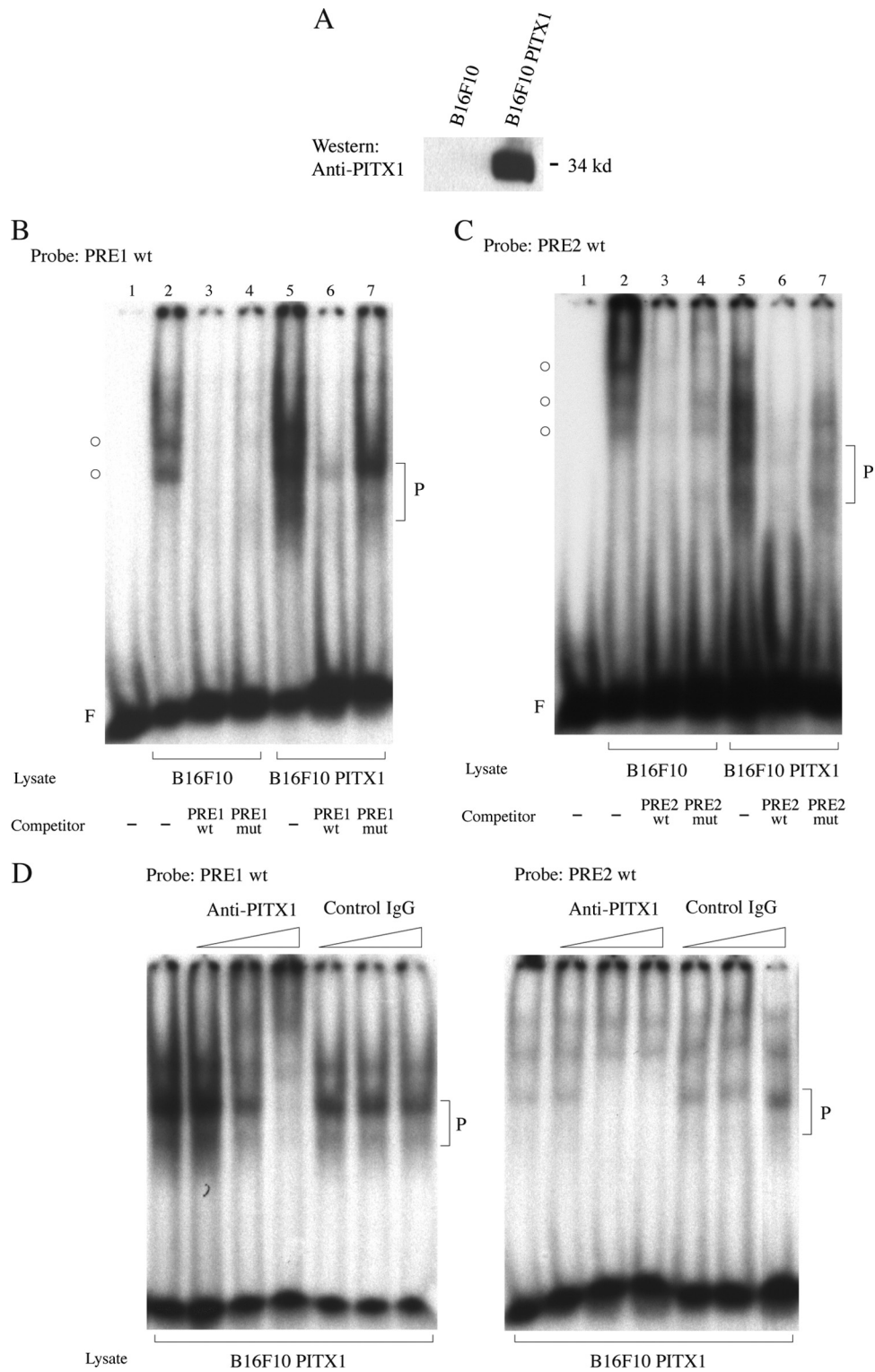


FIG. 6. *PITX1* directly binds to the *hTERT* promoter *in vitro*. (A) Western blotting to confirm the overexpression of *PITX1* protein in B16F10-transfected cells. (B and C) EMSA was performed using the human *PITX1* protein and a radiolabeled oligonucleotide probe designed to detect the binding of *PITX1* to PRE1 (B) or PRE2 (C). The open circles indicate background signals. P indicates probe binding to *PITX1*. F, free probe; wt, wild type; mut, mutation; -, no competitor. (D) A supershift assay using the *PITX1* antibody or control IgG. Triangles indicate increasing amounts of antibody.

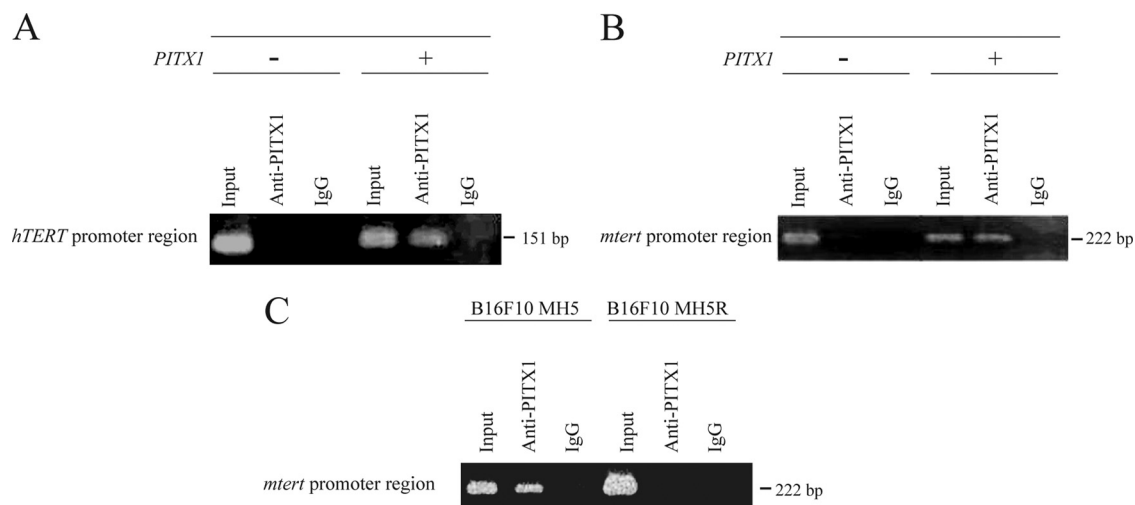


FIG. 7. *PITX1* directly binds to both the *hTERT* and the *mtert* promoter *in vivo*. A ChIP assay was carried out using the anti-*PITX1* antibody to verify the binding of *PITX1* to the *hTERT* and *mtert* promoters in human A2058, mouse B16F10 cells, and the B16F10MH5 clone. *PITX1* expressed in human- and mouse-transfected cells and B16F10MH5 clone was cross-linked to DNA using an anti-*PITX1* antibody. Rabbit immunoglobulin (IgG) was used as a negative control. Input represents PCR of the *hTERT* promoter DNA before immunoprecipitation.

expression of the *hTERT* transgene from a bacterial artificial chromosome containing the entire *hTERT* gene that was introduced into a mouse also was more similar to that of endogenous *hTERT* in humans than to that of endogenous *mtert* (19). Consistently with these findings, our results provide further evidence that the activity of the *hTERT* promoter is under tighter control than that of *mtert*, even in mouse cancer cells (Fig. 4A). These results suggest that specific transcriptional inhibitors in human cells are very important for the strict control of the *hTERT* promoter.

It is known that several elements that are binding sites for transcriptional activators and inhibitors are conserved between the *hTERT* and the *mtert* promoters. In particular, two E boxes and three GC boxes are extremely similar between *hTERT* and *mtert* (Fig. 4B). These boxes lie within the proximal core promoter that is responsible for essential transcriptional activity (33, 49). In contrast, the 5'-flanking sequence of the core promoter is remarkably different between human and mouse (49). Interestingly, the overexpression of the transcription factor activator protein 1 (AP-1) was reported to suppress *hTERT*

transcription by directly binding to two sites on the distal promoter of *hTERT* that are missing from the *mtert* promoter. Although the *mtert* promoter includes other putative AP-1 binding sites, AP-1 overexpression has no effect on *mtert* expression (49). Additionally, two nonconserved GC-boxes exist in the *hTERT* core promoter (Fig. 4C), only one of which was identified as a human-specific repressive element of the *Sp1/Sp3* complex (19). This box is located next to the transcription start site. In the present study, we found a promoter region that was highly conserved between *hTERT* and *mtert* promoters. Of the three candidate *PITX1* binding sites (PRE1, PRE2, and PRE3), only PRE3 exists within this conserved *hTERT* and *mtert* promoter region (Fig. 4B and E). The functional analysis of the *TERT* promoter activity of promoter constructs with mutations in the PRE sites and with various truncations of the promoter region indicated that these promoter disruptions abolished the ability of *PITX1* to repress transcription from the promoter in both human and mouse cells (Fig. 4 and 5). In addition, the inhibition of the complex formed between *PITX1* and PRE1 or PRE2 was observed using an excess of PRE1 and

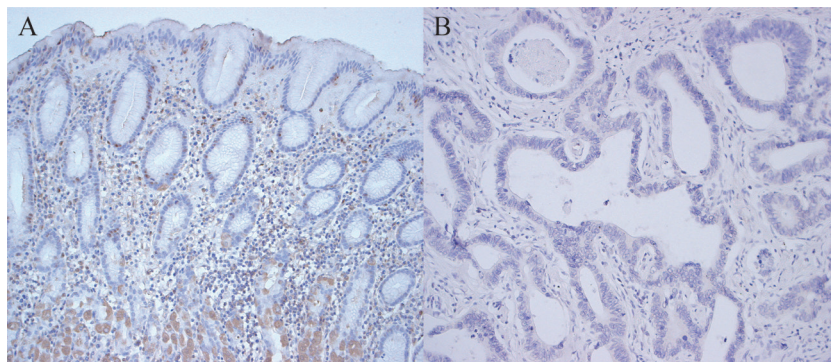


FIG. 8. Detection of *PITX1* protein by immunohistochemistry in gastric mucosa and adenocarcinoma. (A) *PITX1*-positive cells exist in surface mucous cells and fundic glands. (B) In contrast, *PITX1*-positive cells were not observed in carcinoma lesions.

PRE2 probes but not with a PRE3 probe (data not shown) in EMSA, indicating that *PITX1* binds with higher affinity to PRE1 and PRE2 than to PRE3. Thus, these data indicated that the conserved *TERT* promoter region containing PRE sites plays a crucial role in the regulation of *TERT* transcription by *PITX1*, and that the binding affinity of *PITX1* is of different strength for different PRE sites. Further investigation involving a detailed analysis of the *PITX1* affinity of binding to the *TERT* promoter and/or identification of *PITX1* targeting factors may be a great help in understanding the molecular mechanism by which telomerases are differentially regulated in humans and mice.

In accordance with its continued expression in tumor cells, telomerase expression also is maintained in most stem cells, as it is required for the self-renewal ability of stem cells. However, *hTERT* expression is strongly suppressed during differentiation, which contrasts sharply with the moderate downregulation of *mtert*. A recent study has provided evidence that the chromatin environment is a crucial factor for the tight regulation of *hTERT* transcriptional activity in human somatic cells (52). Endogenous *hTERT* and its downstream gene locus, but not the *mtert* gene, are located in a condensed chromatin domain in differentiated cells (52). That study also indicated that condensed chromatin could cooperate with transcriptional repressors on the promoter to effectively reduce the expression of *hTERT* during cell differentiation. It will be of interest to examine if *hTERT* suppression by *PITX1* is involved in stem cell differentiation and to determine the relationship between *PITX1* regulation and chromatin status.

In summary, we have provided evidence that MMCT studies incorporating chromosome mapping by positional cloning strategies, combined with expression profiling analysis, are an effective approach for the identification of tumor suppressor genes, such as negative regulators of telomerase. Our study demonstrated that *PITX1* is a negative regulator of telomerase through the transcriptional repression of the *TERT* promoter. However, the mechanism by which the function of *PITX1* contributes to the transcriptional silencing of the *TERT* gene remains to be clarified. Therefore, future studies that include the functional analysis of proteins or genes that associate with *PITX1* should facilitate our understanding of the molecular mechanisms that are involved in telomerase-dependent senescence or in cancer development.

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